Displacement of protein-bound aptamers with small molecules screened by fluorescence polarization

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Small molecule inhibitors of proteins are invaluable tools in research and as starting points for drug development. However, their screening can be tedious, as most screening methods have to be tailored to the corresponding drug target. Here, we describe a detailed protocol for a modular and generally applicable assay for the identification of small organic compounds that displace an aptamer complexed to its target protein. The method relies on fluorescence-labeled aptamers and the increase of fluorescence polarization upon their binding to the target protein. The assay has high Z′-factors, making it compatible with high-throughput screening. It allows easy automation, making fluorescence readout the time-limiting step. As aptamers can be generated for virtually any protein target, the assay allows identification of small molecule inhibitors for targets or individual protein domains for which no functional screen is available. We provide the step-by-step protocol to screen for antagonists of the cytohesin class of small guanosine exchange factors.

INTRODUCTION

Guanine nucleotide exchange factors (GEFs) constitute a large and diverse family of proteins that facilitate the exchange of bound GDP for GTP on small G-proteins and thus activate them[1–4]. Their pivotal role as G-protein activators makes them attractive targets for modulating G-protein-regulated signaling networks implicated in the establishment of pathological conditions[5–7]. GEF inhibitors, therefore, are of interest both as tools for elucidating the roles of these proteins and their effectors in various disease-related cellular signaling networks and for therapeutic intervention. So far, however, only one small molecule GEF inhibitor, the fungal macrocyclic lactone, brefeldin A (BFA), has been described[8–11]. Structural data indicate that strategies for identifying GEF inhibitors are potentially hampered by the complex kinetics of the multistep GDP/GTP exchange reaction. Starting as a low-affinity G-protein-GDP-GEF complex, the reaction proceeds to form a high-affinity nucleotide-free intermediate to which GTP eventually binds to yield the active G-protein-GTP form[12–14].

A possible strategy for obtaining GEF inhibitors is the evolution of GEF-binding aptamers from extremely diverse libraries of RNA molecules that fold into intricate globular structures. Aptamers are small nucleic acids that can be easily generated by *in vitro* selection of nucleic acid libraries of up to 10^{15} members, the highest diversity currently amenable to screening[15]. The advantage of aptamers is that they can be isolated entirely *in vitro* within short time scales and without specific knowledge of the target's three-dimensional structure, and can bind to and inhibit diverse protein classes with extraordinary potency and specificity[15]. So far, RNA aptamers have been described only for two members of one class of GEFs[17,18], cytohesins 1 and 2.

Cytohesins are GEFs for the Ras-like GTPases of the ADP ribosylation factor (ARF) family[19,20], which control a wide variety of cellular regulatory networks ranging from vesicle biogenesis in intracellular traffic to signaling. Consequently, cytohesins participate in the regulation of diverse processes such as cytoskeletal organization[17], integrin activation[21] or insulin signaling[22,23]. They contain the following modular protein domains which serve distinct functions: a Sec7 domain bearing the GEF activity, a PH domain mediating recruitment to the plasma membrane by binding to phosphatidylinositides and a coiled-coil domain for protein–protein interactions. Due to their molecular mass of about 47 kDa, cytohesins are designated as small ARF-GEFs to distinguish them from the large ARF-GEFs of about 200 kDa, which also contain a Sec7 domain. The investigation of the functions of small GEFs was severely hampered by the lack of inhibiting small organic molecules. Unlike the large ARF-GEFs, the small ones are insensitive to brefeldin A. The only known inhibitor with specificity for small GEFs is M69, an RNA aptamer that binds to Sec7 domains of cytohesins but not to those of members of the large GEFs[17]. *In vitro*, M69 inhibited guanine nucleotide exchange, and its intracellular expression in T cells specifically reduced adhesion to intercellular adhesion molecule 1. This and other reports[18,24,25] demonstrate the potential of aptamers as intracellular inhibitors. However, the main obstacle to achieving broadly applicable inhibition of target proteins in cells, tissues and whole organisms by aptamer technology is transmembrane delivery. For such purposes, a drug-quality small molecule would be highly advantageous compared with any nucleic acid- or biopolymer-based inhibitor, both as a research tool and as a starting point for drug development. A chemical compound can be applied in a spatio-temporally controlled fashion, it often acts transiently and it exerts effects that are reversible. Therefore, different phenotypes can be induced by varying the concentration of the chemical inhibitors. Furthermore, small molecules can target distinct subdomains of a protein rather than affecting a multidomain protein as a whole.

The considerations detailed above sparked the idea of using a known aptamer as a functional template for a small organic inhibitor by developing assays that screen small molecule libraries for compounds that displace the aptamer from its target and are thus likely to reproduce the aptamer's inhibitory activity. The use of highly potent aptamers in displacement screens demands small

molecules of high initial potency, increasing the probability of finding effective and specific inhibitors. The feasibility of such an approach was already demonstrated in our laboratory in successful screens for inhibitors of HIV-Rev[26] and HIV-RT[27]. These screens relied on the construction of reporter ribozymes containing the aptamer domain. These ribozymes were either activated or inhibited upon binding of the cognate protein, and displacement of the protein from the aptamer by a small molecule was detected by a modulation of ribozyme activity. This approach requires elaborate ribozyme design and might prove difficult for laboratories without expertise in the field of nucleic acids.

Here, we describe the detailed protocol for a novel and simple approach to 'converting' a fluorescence-labeled aptamer protein inhibitor into a small molecule with analogous activity. The association of the aptamer with its target is detected by fluorescence polarization. This approach matches the criteria for an excellent screen in being fast (often virtually no incubation time needed), being performed in homogeneous solution and in being highly reproducible with a Z′-factor[28] greater than 0.7. In addition, it is affordable for academic research. Apart from the access to the collections of chemicals to be screened for activity, the only non-standard equipment it requires is a plate reader capable of measuring fluorescence polarization. All required reagents are readily available from commercial sources.

Fluorescence polarization measures the rotational diffusion of a molecule, a parameter that is inversely proportional to the molecular volume and thus, in most cases, indirectly to the molecular weight of a fluorescence-labeled complex. Fluorescence polarization (P) has been used for the detection of a variety of molecular interactions, for example, between proteins[29], protein and nucleic acids[30], and receptor and ligand[31].

After excitation with planar polarized light, a fluorophore emits fluorescence in the same plane of polarization. P is quantified (see equation (1)) as the difference in the intensity of emitted light in the polarization plane and in the plane perpendicular to it from both planes of polarization divided by the total intensity of emitted light.

$$P = \frac{I_{II} - I_I}{I_{II} + I_I} \quad (1)$$

In equation (1), I_{II} is the intensity of the emitted light parallel to the plane of excitation, and I_I is the intensity of emitted light perpendicular to the plane of polarization.

Due to molecular motion of the fluorophores in the excited state, however, there is a degree of depolarization of the emitted light measured. This depolarization effect depends on the size of the fluorescent complex, the lifetime of the fluorophore and the viscosity of the solution. The dependence of fluorescence polarization on these parameters is summed up in Perrin's equation (2).

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{\tau}{\phi}\right) \quad (2)$$

$$\text{where } \phi = \frac{\eta V}{RT}$$

In equation (2), P_0 is the limiting polarization of the fluorophore (without rotation), τ is the lifetime of the excited state, η is the viscosity of solvent, V is the molar volume of fluorescent complex, R is the gas constant and T is the temperature.

In our assay, the aptamer is fluorescence-labeled at its 5′ terminus with a fluorescein dye via a C_6-linker. A sulfhydryl group is introduced at the 5′ terminus of the aptamer by incorporation of guanosine monophosphothioate (GMPS) as a starter nucleotide[32]. This modification introduces a nucleophilic residue into the aptamer that can then easily react with iodoacetamido compounds[33]. **Figure 1** schematically shows the (ideal) aptamer replacement scenario on which the present assay is based. In its unbound state, the labeled aptamer exhibits low polarization (black bar to the left of the graph). When the aptamer is bound to the target protein, the fluorescence polarization increases (gray bar, middle) due to the larger molecular volume of the complex. If a small molecule displaces the aptamer from the protein, fluorescence polarization decreases again (black bar, right). If the aptamer–protein complex is incubated with a library of small molecules, those molecules that disrupt the binding between the aptamer and protein can be identified by the reduction in the polarization to the level of aptamer alone.

It is imperative that the aptamer does not lose the ability to specifically recognize the target protein upon derivatization with the fluorophore. If the 5′ end of the aptamer is necessary for specific protein binding, the 3′ end or suitable internal sites within the sequence can be used for coupling the fluorescence label[34]. The linker attaching the fluorophore to the aptamer needs to be as short as possible to prevent unwanted depolarization due to the so-called 'propeller effect'[35]. It is also important in this assay that the molecules in the library do not display fluorescence in the same wavelength region as the fluorophore of choice. In this case, in fact, the fluorescence polarization observed would be a superposition of the polarization of the aptamer and the polarization of the small molecule.

It is interesting to note the consequences of Perrin's equation for aptamer displacement screening assays by fluorescence polarization. In equation (2), ϕ is directly proportional to V, the molar volume of the fluorescent complex. Thus, the larger the difference between the mass of the aptamer and that of the protein, the larger the polarization difference (ΔP) (and, hence, the better the assay quality as quantified by the Z′ value; see equation (3) below). This means, for example, that a small fluorescence-labeled

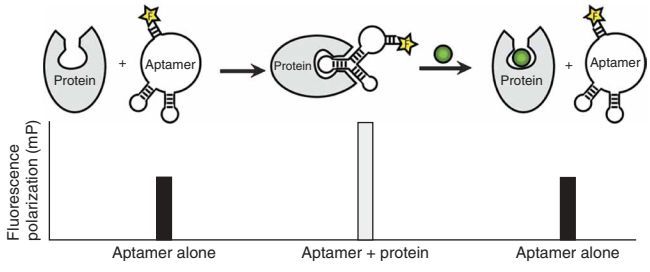

Figure 1 | Schematic representation of the ideal scenario probed by the aptamer-displacement screening assay by fluorescence polarization. The fluorescence-labeled aptamer exhibits low polarization (black bar, left) in the non-bound state. When bound to the target protein, the fluorescence polarization increases (gray bar, middle). If a small molecule displaces the aptamer from the protein, the fluorescence polarization decreases (black bar, right).

aptamer bound to a large protein target will result in a considerably higher value of ΔP than a larger aptamer bound to a smaller protein.

$$Z' = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|} \quad (3)$$

In equation (3), σ is the standard deviation of the positive (p) and the negative (n) control, μ is the mean value for polarization (for positive (μ_p) and negative (μ_n) controls).

We applied the present method in the screening of small molecules inhibiting the cytohesin family of small GEFs[22] using M69, an aptamer specific for cytohesin Sec7 domains that inhibits GEF activity on ARF proteins. We used a library of up to 10,000 structurally diverse compounds that comprised chemotypes of different scaffolds, adhered to 'Lipinski rule-of-five'[36] and had molecular weights below 600 Da. To make sure that the compounds do not interfere with the polarization assay, we determined their fluorescence in the fluorescein wavelength range and eliminated those that showed fluorescence. The best of the 20 hits identified, SecinH3, exhibited similar properties as the parent aptamer. It binds specifically to all members of the cytohesin family, across different species, with a dissociation constant of about 250 nM, and it inhibits the GDP/GTP exchange on ARF proteins in cultured cells and *in vitro* with an IC_{50} of about 5 μM. We demonstrated the potential of the use of SecinH3 in chemical genetics by applying it to a cultured liver cell line, HepG2, and *in vivo* by feeding it to mice. By the application of SecinH3 we could prove that cytohesins are a part of the insulin receptor complex and are fundamental for the insulin signaling pathway, and that their inhibition *in vivo* results in hepatic insulin resistance[22,23].

Our method is generally applicable to the screening of small molecule libraries for inhibitors of proteins for which either aptamers or natural nucleic acid binders are known. A number of different selection strategies for aptamers have been described[37–39]. The small molecules found are promising pharmacophores that can be developed into lead structures and potential drugs.

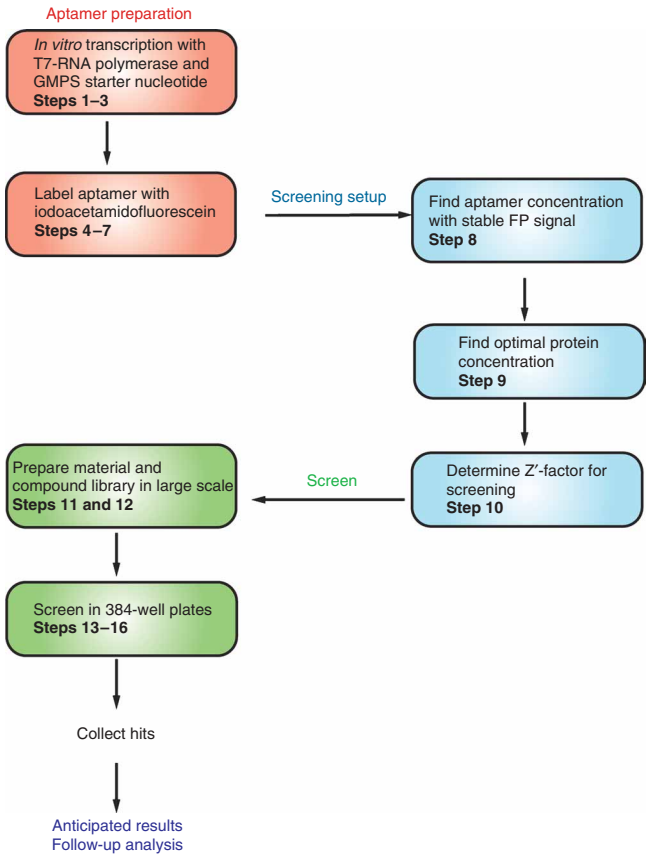

Figure 2 | Workflow for the setup and execution of a fluorescence-polarization screen. The numbers refer to the numbered steps in the step-by-step protocol.

Our detailed protocol consists of three parts (see **Fig. 2**). The first part (Aptamer preparation) describes the *in vitro* transcription and fluorescence labeling of the aptamer. Next, the optimal screening conditions are established to ensure high selectivity and sensitivity (Screening setup), before the 'Screen' itself can be performed. An overview of the follow-up analysis is presented in ANTICIPATED RESULTS.

MATERIALS

REAGENTS

- GMPS (EMP Biotech)
- 5-Iodoacetamidofluorescein (5-IAF; Sigma, cat. no. I9271)
- Sodium acetate (Merck)
- Ethanol (Fluka)
- T7-RNA polymerase (Stratagene, cat. no. 600123)
- RNase inhibitor, RNasin (40 U μl^{-1}; Promega)
- dsDNA of M69 aptamer bearing a T7 promoter sequence (cytohesin aptamer M69—template sequence: 5′-GGGAGAGACAAGCTTGGGTCTATTATGC CTTTAGCTAGCGCATTCTGTGGGGTGGGTGGAAGAAGAGAAAGAGA AGTTAATTAAGGATCCTCAG-3′; forward primer: 5′-*TCTAATACGACTCA CTATA*GGGAGAGACAAGCTTGGGTC-3′ (T7 promoter sequence is italicized); reverse primer: 5′-CTGAGGATCCTTAATTAACTTCTCT-3′)
- MicroSpin gel filtration G-25 columns (GE Healthcare)
- Urea (Calbiochem)
- Tris-HCl (Roth)
- Tris/EDTA (TE) buffer
- Dimethyl formamide (DMF), p.a. (Fluka)
- Diethylpyrocarbonate (DEPC; Sigma) ! **CAUTION** Carcinogenic, handle in fume hood.
- Dimethyl sulfoxide (DMSO) for cell culture (Sigma)
- Phosphate-buffered saline (PBS)
- 1 M $MgCl_2$ (Merck)
- NaCl (Merck)
- NTPs (ATP, GTP, UTP, CTP), 100 mM solution (Roche)
- Sec7 domain of cytohesin-1, recombinantly expressed in *Escherichia coli* and purified on Ni-NTA-agarose
- Compound library dissolved in DMSO, 1 mM concentration
- Dithiothreitol (DTT, 100 mM)
- Spermidine
- Inorganic pyrophosphatase (IPP, 2 U μl^{-1}; Roche)
- Acrylamide/bis-acrylamide (Roth) ▲ **CRITICAL** Make sure all the reagents mentioned above are RNase free.

EQUIPMENT

- Tecan Ultra microtiter plate reader (MTP) equipped with appropriate excitation, emission and polarization filters (for fluorescein excitation, 485 nm; absorbance, 520 nm) or any other microplate reader capable of reading fluorescence polarization
- 384-well plates, black (Greiner)
- 96-well plates, round-bottomed with cover, for aliquoting the compound library
- Electronic multichannel pipettes, 100 and 10 μl (Eppendorf)

- Liquid-handling workstation (optional and depending on the library size; Freedom EVO, Tecan)
- PAGE equipment for denaturing polyacrylamide gels (15 cm × 17 cm): glass plates, gel chamber (Bio-Rad) and power supply (Consort E865)
- Sterile scalpels
- Siliconized glass wool
- Cooled microcentrifuge (Eppendorf 5814C)
- UV spectrophotometer (Thermo Life Sciences)
- UV hand lamp
- TLC plates silica gel 60 F254 20 cm × 20 cm (Merck, cat. no. 105805) for UV shadowing
- Plastic wrap

REAGENT SETUP

DEPC-treated PBS Add 1 ml of DEPC per liter of PBS, stir for at least 2 h and autoclave. ! **CAUTION** DEPC is carcinogenic until autoclaved; use gloves and work in a fume hood. ▲ **CRITICAL** Unreacted DEPC modifies nucleic acids. It is critical to deactivate the DEPC by thoroughly autoclaving.

DEPC-treated water Add 1 ml of DEPC per liter of distilled water, stir for at least 2 h and autoclave. ! **CAUTION** DEPC is carcinogenic until autoclaved; use gloves and work in a fume hood.

5-IAF Prepare a 10 mM stock solution of 5-IAF in DMF. ▲ **CRITICAL** Protect this stock solution from light by wrapping the container in aluminum foil or using darkened glass vials. Prepare freshly before using it for the labeling reaction.

EQUIPMENT SETUP

Liquid-handling workstation Using a liquid-handling workstation often results in better Z′ values compared to manual pipetting using the electronic multichannel pipette. In screening assays with borderline Z′ values (0.5–0.55) we recommend use of the liquid-handling workstation.

PROCEDURE

Fluorescence labeling of the aptamer ● TIMING 1 d

1| Prepare the dsDNA template of the aptamer that is used in the *in vitro* transcription reaction in a standard polymerase chain reaction, and purify it according to conventional molecular biology protocols.

2| Perform an *in vitro* transcription of the M69 DNA in the presence of GMPS to obtain 5′-thioate-labeled RNA. As neither self-prepared nor purchased GMPS is free of RNases, it is critical to add RNase inhibitors. Set up the *in vitro* transcription reaction as follows:

Reagent	Concentration
40 mM Tris (pH 7.9)	1×
NTP mix (25 mM each)	2.5 mM
RNasin (40 U μl^{-1})	0.4 U μl^{-1}
$MgCl_2$ (100 mM)	25 mM
DTT (100 mM)	10 mM
GMPS	20 mM
dsDNA template (from PCR)	100 pmol
T7-RNA polymerase (25 U μl^{-1})	2.5 U μl^{-1}
H_2O	To give 100 µl final volume

Incubate the reaction mixture overnight at 37 °C.

3| Perform a standard phenol/chloroform extraction and ethanol precipitation (for detailed information on general handling of nucleic acids, see ref. 40) of RNA, dissolve the pellet in 100 µl H_2O and perform two consecutive G25 gel filtrations according to the instructions of the manufacturer.

▲ **CRITICAL STEP** The gel filtrations are necessary to remove the DTT and unreacted GMPS, whose free thiol groups can react with the IAF.

▲ **CRITICAL STEP** To minimize hydrolysis, the RNA solution is stored frozen at −20 °C or below, and kept on ice while reactions are being set up.

4| Prepare the following fluorolabeling reaction mixture:

Reagent	Concentration
GMPS-RNA	100 µl
TE (10×)	1×
Urea (8.3 M)	2 M
RNasin (40 U μl^{-1})	0.4 U μl^{-1}
H_2O	To give 400 µl final volume

5| Heat the reaction mixture obtained from Step 4 to 80 °C for 3 min and add 100 µl of a 10 mM solution of freshly dissolved 5-IAF in DMF. Incubate for 2 h at 37 °C while protecting the mixture from light.

6| Purify the reaction product by denaturing PAGE. A 10% (wt/vol) polyacrylamide gel was used for the aptamer M69, which is 94 nt long. Please note that for shorter nucleic acids it may be appropriate to use a gel of a higher percentage of polyacrylamide. Try to avoid exposure to light during electrophoresis. Wrap the gel in plastic to protect the RNA and visualize the RNA bands on

the gel by UV shadowing on a TLC plate (containing Fluorosil) with a UV hand lamp. Cut out the band of the anticipated length with a sterile scalpel, crush it and resuspend it in three volumes of 0.3 M sodium acetate (pH 5.4). Shake the suspension for 1 h at 65 °C and then filter it through siliconized glass wool. This purification step removes shorter RNAs generated in the *in vitro* transcription reaction. The gel resolution is generally not high enough to separate the unlabeled aptamer from the fluorescence-labeled aptamer. However, this separation is usually not necessary because of the high efficiency of the labeling reaction.

7| Precipitate the RNA by adding three volumes of 100% ethanol and cooling to −80 °C for 10 min. Spin at the maximum speed for 20 min in a microcentrifuge at 4 °C; wash the pellet with 70% ethanol and centrifuge again for 5 min at maximum speed (20,000*g*) at 4 °C. Resuspend the dry pellet in 30 µl water. Determine the concentration of RNA by UV spectrometry using the following equation:

$$c_{RNA} = \frac{[A_{260} - (A_{492} \times 0.35)]D}{\varepsilon_{RNA}} \quad (4)$$

$$\left(\frac{RNA_{fluorescein\text{-}labeled}}{RNA_{total}}\right) = \frac{A_{492} D\, MW_{RNA}}{\varepsilon_{fluorescein}\, c_{RNA}} \quad (5)$$

The extinction coefficient for RNA (ε_{RNA}) is the sum of the individual extinction coefficients of the bases (ε_A (mM^{-1} cm^{-1}) = 15,200; ε_C = 7,050; ε_G = 12,100; ε_U = 8,400). The extinction coefficient of fluorescein ($\varepsilon_{fluorescein}$) at 492 nm is 73,903 mM^{-1} cm^{-1}. *D* is the dilution factor, *A* is the absorption and MW is the molecular weight.

The overall yield at this point should be approximately 3 nmol of purified, fluorescence-labeled M69. Please note that for the screening it might be necessary to scale up the reaction.

■ **PAUSE POINT** The fluorescence-labeled RNA can be stored in the dark at −20 °C for at least 1 month.

? TROUBLESHOOTING

Screening setup ● TIMING 1 d

8| To find the optimal aptamer concentration that gives a stable polarization signal, pipette a dilution series of 50–250 nM fluorescence-labeled aptamer in PBS (pH 7.4) and 3 mM $MgCl_2$ in a 50 µl reaction volume, and determine the fluorescence polarization. Please note that, when using the Tecan Ultra MTP, you will find that the polarization increases with decreasing aptamer concentrations, which is a measurement artifact due to decreasing fluorescence intensity. Other fluorescence readers may show other behavior at fluorescence intensities near the detection limit. In general, screening is carried out in the selection buffer for the aptamer. Bear in mind that the fluorescent properties of the fluorophore depend on the pH of the buffer.

▲ **CRITICAL STEP** It is essential that polarization at the aptamer concentration which you will use for screening is measured at an aptamer concentration that gives a stable polarization signal, that is, a concentration level at which a further increase in aptamer concentration does not result in polarization change.

9| Find the optimal protein concentration for which the signal-to-noise ratio is maximum. Start with an aptamer-to-protein ratio of 1:1, 1:5, 1:10 and 1:20 and determine the polarization difference between aptamer alone and the aptamer–protein complex. Add DMSO to a final concentration of 10% to the reaction mixture, as the compound libraries are dissolved in DMSO. Screen at the minimal possible protein concentration at which the polarization of the aptamer–protein complex is maximal. As a control, determine the polarization difference for the binding of the aptamer to an unrelated protein, such as BSA. In this case, in fact, the measured polarization should be the same as that of the aptamer alone.

? TROUBLESHOOTING

10| Determine the Z′-factor for the screening setup (see equation (3)). For this, determine the polarization difference between the aptamer and the aptamer–protein complex by determining the fluorescence polarization of at least an eightfold replicate for each of the two reaction mixtures. If the Z′-factor is above 0.5, the assay should be stable enough to start screening with a limited number of compounds. If you intend to screen >25,000 compounds, the Z′-factor should be above 0.7.

■ **PAUSE POINT** At this point the screen is set up. The next step is the actual large-scale screen itself.

Screen ● TIMING ~1 h per 100 compounds

11| Ensure that you have prepared enough fluorescence-labeled aptamer and protein for the number of compounds you intend to screen. For instance, for the screening of 5,000 compounds with M69 and the Sec7 domain of cytohesin-1, about 120 nmol of fluorescence-labeled M69 and 1.2 µmol (ca. 34 mg) of the Sec7 domain were needed (see ANTICIPATED RESULTS).

12| To make the screening amenable for multichannel pipettes or a pipetting robot, prepare the compound library in a high-throughput friendly format by aliquoting the compounds dissolved in DMSO into 96-well plates at a 1 mM concentration. Leave the last column (column 12) in each row of the 96-well plate free of compounds and add only DMSO for the controls. Thus, each plate contains 88 small compounds.

13| For each compound plate to be screened, prepare two solutions, one with and one without the target protein, as follows:

	Solution 1	Solution 2	(Stock)	(Final)
M69	2 ml	2 ml	500 nM	100 nM
Sec7	–	2 ml	5 μM	1 μM
Buffer (5× PBS, 15 mM $MgCl_2$)	2 ml	2 ml	5×	1×
H_2O	5 ml	3 ml		

14| The screening can be performed in 96-well or 384-well plates. For each compound to be screened, determine, in duplicate, the polarization of the aptamer in the presence and absence of the target protein. For this purpose, pipette 5 μl of compound from the compound plate in quadruplicate into a 384-well plate (or a 96-well plate). To maximize time efficiency in the semiautomatic assay, it is most convenient to dispense the compound from well A1 in the library plate into wells A1, B1, A2 and B2 of the sample plate (making a 2 × 2 square), the compound from well A2 into wells A3, B3, A4 and B4, etc. Add 5 μl of DMSO into the wells of the last two columns of a 96-well plate or the last two columns of a 384-well plate (columns 23 and 24 for a 384-well plate and 11 and 12 for a 96-well plate) for the controls.
▲ **CRITICAL STEP** Make sure not to cross-contaminate the compounds in the library plate. Change the pipette tips if you are using disposable tips or thoroughly rinse the tips if you are using a pipetting robot with needles.

15| Dispense 45 μl of solution 1 into the odd-numbered columns of the plate (columns 1, 3, 5,..., 23) and 45 μl of solution 2 into the even-numbered columns (columns 2, 4, 6,..., 24).

16| Shake vigorously using the shaking function of the MTP reader, incubate for 10 min at 37 °C and measure the fluorescence polarization. Calculate the Z′-factor for each plate using the polarization values from the control columns 23 and 24 (11 and 12 for a 96-well plate). If the Z′-factor is significantly below the one obtained in Step 10, rescreen the plate, because the data obtained will not be trustworthy. Calculate the mean values and the standard deviations of polarization in the presence and absence of the target protein for each compound.

● **TIMING**
For Steps 1–3: approximately 45 min per plate of 88 compounds in our semiautomated setup. The number of compounds processed on one plate can easily be scaled up by using 1,536-well plates and reducing the reaction volume to 10 μl. A good Z′ value indicates a robust and stable screen, therefore it is not necessary to measure in duplicate, further reducing the screening time. In a semiautomatic screen within an academic setting, it is feasible to screen approximately 780 compounds in approximately 1 h.

? TROUBLESHOOTING

Step 7
If the yield of labeled RNA is very low, you might have a problem with RNases. Take care to work in an RNase-free environment. RNases can be introduced by the operator at any point of this protocol, and it may lead to severely reduced yield or total loss of RNA. Degraded RNA can be detected by UV shadowing of the polyacrylamide gel as a smear of lower molecular weight below the RNA of correct length. Furthermore, the fluorescence-labeling method using GMPS *in vitro* transcription followed by chemical derivatization by 5-IAF rarely exceeds 50% efficiency[41]. Thus, in some cases where the fluorescence intensity is very low and therefore you need a high aptamer concentration to get a stable polarization signal, it might be advantageous to use an aptamer probe that is produced entirely by oligonucleotide synthesis, including the fluorescent tag.

Step 9
If the aptamer binds to unrelated proteins, consider adding unspecific competitors like salmon sperm DNA, tRNA or heparin to the reaction mixture. Perform Step 9 again to find the concentration of competitor that gives the optimal polarization difference while disrupting any unspecific binding of the aptamer.

If a polarization difference between the aptamer and aptamer–protein complex is not obtained, the aptamer might not be functional, for example, because the bulky fluorophore blocks the interaction between the aptamer and protein. Check for this possibility by determining the dissociation constant (K_D) of the fluorescence-labeled aptamer by filter-binding or surface

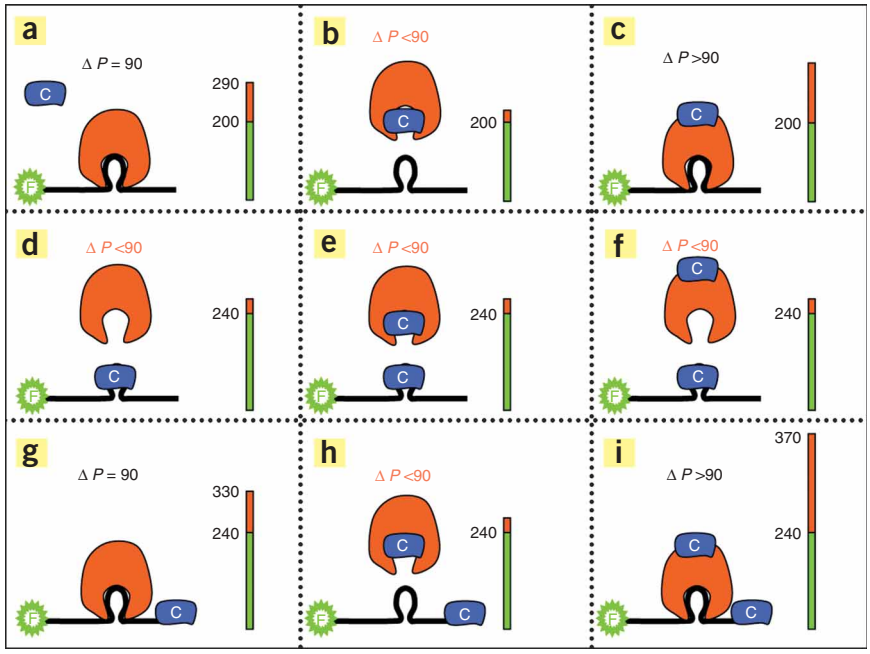

Figure 3 | Nine possible modes of action for the compound in an aptamer-displacement assay system. In the M69/Sec7 system, if the compound (C) binds neither the protein nor the RNA (**a**), the polarization of the aptamer alone (green bar) is approximately 200 mP, the polarization value of the aptamer–protein complex is approximately 330 (orange bar) with a polarization difference ΔP of approximately 90 mP. A compound that displaces the aptamer from the protein by binding to the protein (**b**) reduces the polarization of the aptamer–protein complex and ΔP decreases. If binding of the compound to the protein does not lead to displacement of the aptamer (**c**), the polarization difference might increase slightly. Compounds that bind to and change the folding of the RNA increase the polarization of the aptamer alone (**d**–**f**) (here to about 240 mP) but would be detected as hits if only the polarization difference is considered. Other possible modes of binding include interaction of the compound with the aptamer without displacement of the protein (**g**), and unspecific interaction with the protein and the aptamer (**h** and **i**).

plasmon resonance. If the K_D is considerably higher than that of the unlabeled aptamer, consider another labeling method, such as 3′ labeling[34], or introducing the fluorescent tag by oligonucleotide synthesis into positions that are not necessary for protein binding. Also, make sure that the protein itself is functional in an *in vitro* assay, if possible, or that it folds properly so that the underivatized aptamer can recognize it in a filter-binding assay.

If a low polarization of the aptamer alone and a low polarization difference of the aptamer–protein complex are obtained, consider adding glycerol to increase the viscosity (see Perrin's equation (2)) of the reaction mixture. Perform Step 9 again.

ANTICIPATED RESULTS

The parameters to be measured are the polarization of the aptamer in the presence of the compound and the polarization of the aptamer in the presence of the compound and the target protein. A compound that completely displaces the aptamer from the protein causes the measured polarization to revert to that of the aptamer alone (negative control). Hence, all compounds that reduce the polarization difference can be regarded as potential hits. There are, however, several other modes of interaction between the compound and the aptamer–protein complex that can reduce the polarization difference and yield false-positive hits (see **Fig. 3**). Most of these interactions involve the binding of the small molecule to the aptamer itself. Of the small molecules binding to the aptamer, especially those that change the molecular volume of the aptamer will be picked up efficiently by fluorescence polarization (see Perrin's equation (2)). These compounds can be excluded from further investigation by comparing the polarization of the aptamer alone, with and without the small molecule. Only those compounds that do not

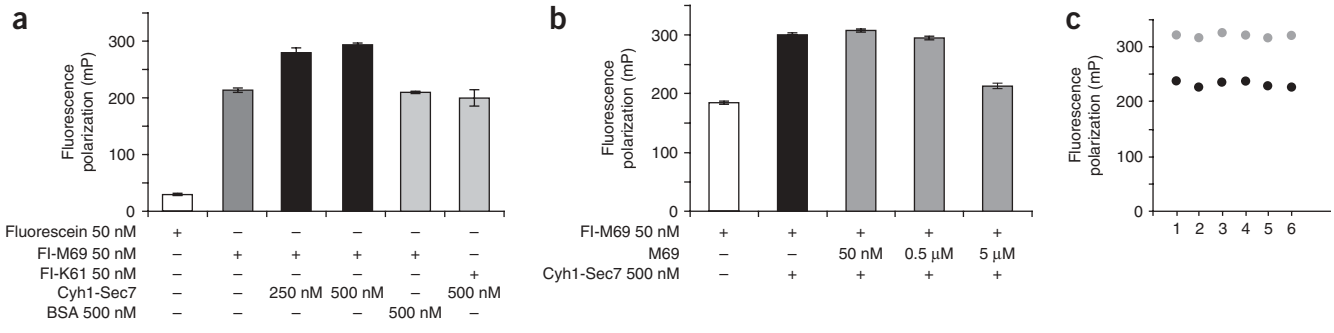

Figure 4 | Screening setup to use M69 in a robust aptamer-displacement assay to screen for inhibitors of cytohesin (Cyh)1-Sec7. (**a**) Fluorescence polarization of labeled aptamer M69 (dark gray bar) increases specifically upon binding to Cyh1-Sec7 (black bars). Controls: M69 does not bind 500 nM BSA and labeled K61, an aptamer of similar size as M69 that is specific for cytohesin-2 and does not bind Cyh1-Sec7 (light gray bars)[18]. (**b**) Unlabeled M69 displaces fluorescence-labeled M69 from Cyh1-Sec7. (**c**) The Z′ value was calculated from eight independent measurements of the polarization difference shown here. From these values, a Z′ of 0.7 was calculated.

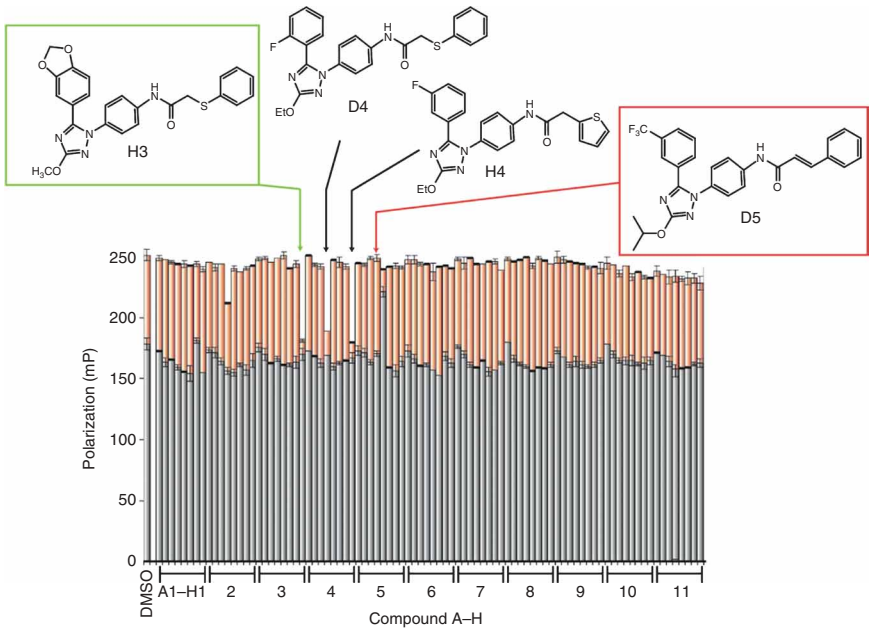

Figure 5 | Representative example of the screening results of a box of 88 compounds with the structures of three hits indicated. Orange bars: fluorescence polarization level of the aptamer M69 in the presence of cytohesin-1-Sec7 and compounds[22]. Gray bars, control measurements in the absence of protein. All of these hits inhibited cytohesins *in vitro* and in cell culture. The compound D5 was selected as a negative control for the follow-up experiments because of its structural similarity with SecinH3 (H3).

alter the aptamer's polarization in the absence of the target protein should be considered further. We excluded compounds that seemed to interact with the RNA (**Fig. 3e–h**) because we were interested in compounds with as little potential unspecificity as possible, as they were to be tested *in vivo*. As a first step of further characterization, K_D can be estimated by measuring the polarization difference with increasing concentrations of the compound.

In our screening system, we first established the specificity of the interaction of the fluorolabeled aptamer M69 with cytohesin-1-Sec7. We showed that the polarization of the aptamer rises with increasing amounts of protein in a dose-dependent manner (**Fig. 4a**), whereas the polarization level is not affected by BSA, a protein that the unlabeled aptamer does not interact with. To rule out any unspecific interaction between the fluorophore and cytohesin-1-Sec7, an aptamer that does not bind cytohesin-1 was labeled and incubated with the protein, and polarization data confirmed that no interaction took place. To verify that the fluorolabeled aptamer binds to the same epitope on the protein and that it is possible to displace the aptamer (M69), a titration experiment with an excess of unlabeled M69 was performed (**Fig. 4b**). The Z'-factor of 0.7 determined in Step 10 indicated that the screen is robust and reproducible (**Fig. 4c**).

A screen of a library of approximately 10,000 diverse compounds was performed[22] (**Fig. 5**). A hit was defined as a compound that reduced the polarization difference of the aptamer by more than 50%. Follow-up analysis by surface plasmon resonance showed that 20 of the obtained hit compounds bound to cytohesin-1 with good affinity (low micromolar K_D). Most, but not all of these compounds, also inhibited the GEF function of cytohesin-1. Hence, the described method yields a manageable amount of compounds as initial hits, out of which several compounds are active (**Fig. 5**).

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